Title

IgY Against Dental Caries and Dental Caries – Preventine Combination

Background of the Present Invention

Field of Invention

The invention is put under preparation of immunoglobulin from hen yolk, especially, IgY against dental caries bacteria and the combination preventing dental caries wherein the IgY and antiseptic are as effective components.

Description of Related Arts

It is well know that streptococcus mutans are major dental caries bacteria. There are two measures of passive immunization to streptococcus mutans at the moment. First, cows or hens are immunized with single or mixed streptococcus mutans as antigen, the antibodies are extracted from milk or yolk, then passive immunization is taken place. Second, cows or hens are immunized with glucosyl transferase of streptococcus mutans as antigen, the following steps are as same as those of the first. There are, however, problems for available preparative technique of antibody: 1. high cost of antibody preparation, especially, difficulty of antigen extract while glucosyl transferasse is used as antigen. 2. low titer of antibody, the highest titer is only 1:320 by existing preparative technique. 3. The yolk could not be comprehensively used.

Summary of the Present Invention

The purpose of present invention raises a preparative method of IgY against dental caries, which reduces cost of production, elevates titer of antibody, and yolk can be utilized in multi-propose, simultaneously, provides dental caries preventing combinations where IgY of the invention and antiseptic are as effective components.

The preparative method of IgY against dental caries in the invention is as the following:

Streptococcus mutans type c and type d are separately grown BHI or TTY culture medium for 2-3 days centrifuged to collect the bacteria. The bacteria are rinsed 4-6 times with 0.05-0.2 M of phosphate buffered saline, pH 6-7, heated at 50-60°C for 25-35 minutes. To prepare antigens, mix streptococcus mutans type c and type d by ratio 1-2:1, add Freund' s adjuvant equal to total volume of both bacteria, then treated with high speed homogenized.

The best ratio of type c and type d mixture is 2:1.

The hens are immunized by three hypodermic or wing vein injections, 1.0ml (1x10°/ml) of streptococcus mutans each time, at 2weeks intervals. Yolks are taken out by sieve, stirred even, diluted by adding 4-6 fold of distilled water. Adjust pH to 4.5-6.5, stand at 3-5°C for 20-30 hours, centrifuge at high speed for 20-30 minutes. The supernatant is ultra filtrated, followed by sterilization and lyophilization. This is crude IgY extract against dental caries.

The crude extract is applied on DEAE-Sephadex A50 column, eluted with phosphate buffer containing 0.03-0.1M of NaC1 by gradient elution followed by pouring protein peaks, estimating antibody activity with ELISA, and adjusting active eluates to 20mg protein /ml.

Obtained eluates are applied on Sephadex G200 column, eluted with phosphate buffer containing 0.05-0.2M of NaC1 by gradient elution followed by pouring protein peaks, estimating antibody activity with ELISA, sterilizing by 0.22M membrane filtration, and lyophilizing. T is purified IgY against dental caries bacteria.

The best concentrations of NaC1 in phosphate butter eluants for DEAE-Saphadex A50 and Sephadex G200 are 0.07Mand o, 1M, respectively.

Based on the following results, present invention chooses streptococcus mutans type c and type d as antigen bacteria.

Streptococcus mutans have serotype a, b, c, d, e, f, g, and h, etc. in accordance with serum typing. In oral cavity, however, type c type d account for about 60-90%and10%, respectively, the others are very low, therefore, type c and type d are major serotype bacteria causing dental caries. Choosing type c and type d as antigen bacteria advantage in either avoiding reducing effectively of major serotype bacteria when mixture of multi- serotype bacteria is used as antigens, or avoiding narrow range of immunological cross reaction when single serotype bacteria is used as antigens. Moreover, the investigations indicate that protein antigen A and B can be extracted from cell wall of streptococcus mutans of oral cavity, type c has both protein A and B, type b has only protein A type a, d, e, and g have only protein B, thus, type c and d as antigens insure that the antibodies have wide range of cross reaction.

The invention adopts water dilution to extract IgY, which comprehensively utilizes of the yolks with low cost, simple technology and no environmental pollution.

IgY of present invention has reached PAGE purity with 180 kD of molecular weight by SDS-PAGE.

The experiments showed that the IgY keeps its activity during 90 minutes at 65°C; its activity has no significant change after 8 hours at 37°C, but rapidly decreased, then inactivated at pH 2.0 or pH 12.0; the IgY is resistant to osmotic pressure, for instance, tolerant to 40% sucrose.

The IgY of the invention can effectively inhibit agglutination of streptococcus mutans by indirect hem agglutination test with the titer I:51, obviously inhibit adhesion of the bacteria until it is diluted to 1:8; animal experiments takes that IgY can effectively prevent occurrence of dental caries by feeding IgY rats infected with streptococcus mutans, the bacteria number in bacteria plaques reduce 70-80%, the results of contrast experiment of dental caries formation in rats is expressed in the following table:

	Control	Experiment	P Value
I	47	19	< 0.01
II	25	0.57	< 0.01
III	8	0	< 0.01

I: only damage of enamel; II: damaged ¼ of denting; III: damaged through dentine. Dental caries score by Keyes method.

The difference between two groups is very significant ($p < 0.01$).

The dental caries preventing combination means that the combination's effective components are IgY of present invention and antiseptic, the later is, at least, one of both potassium sorbate and sodium benzoate, the combination may be either a product for oral cavity use, for example, toothpaste, buccal liquid, mouthwash, or food, for instance, chewing gum, chocolate, ice cream, milk (powder), bean milk (powder), etc. Additive amount of the IgY usually is 0.05-0.2%, potassium sorbate or sodium benzoate is 0.005-0.02%.

The combination can be packaged in pocket atomizer when as liquid product used in oral cavity, and in sucking bottle as food for serving.

Coordinated with decontaminaters and ozostomia preventer, diverse products can be manufactured of the IgY for prevention and treatment. Also, it can be added in oral cavity frushres to enhance their function preventing dental caries.

The IgY of the invention is low cost of preparation and production, high titer of antibody, resistant to osmotic pressure, strong immunological activity and wide range of cross-reaction to streptococcus mutans. The combination of present invention features in small amount of IgY, safe use, effective prevention and treatment, etc. It can effectively prevent occurrence of dental caries.

Detailed Description of the Preferred Embodiment

The invention will be described further by the following samples:

Sample 1 Streptococcus mutans type c and type d are separately cultivated in BHI medium at 37°C for 48 hours, followed by collecting bacteria with centrifugation, at 4000 rpm for 10 minutes, winsing 5 times with 0.2M of phosphate buffered saline, pH 6.0, and heating at 50°C for 25 minutes. Each of type c and type d is adjusted to 2x10 /ml of the suspensions. Mix equal volume of type c and type d, then, Freund's adjuvant equal to total volume of type c and type d is added in it then, homogenized at high speed. This is a streptococcus mutans antigen.

The hens are immunized by three hypodermic injuctions, 1.0ml 91x10 /ml) of bacteria antigens each time at 2weeks intervals. Eggs are collected from 20th day after first immunization, and sterilized with 75% alcohol. The yolks are taken out with sieve and stired to even, diluted with 5 fold distilled water, adjusted pH to 6.0, standed at 3°C for 24 hours, then, centrifuged at 2000 rpm for 20 minutes. The supernatant is concentrated by ultra filtration, sterilized and lyophilized. This is crude IgY extract against dental caries bacteria.

Three milliliter (10 mg/ml) of crude IgY extract are applied on DEAE – Sephadex A50 column (2.5x35cm), eluted with pH 7.0, 0.01M of phosphate buffer containing 0.07M of NaCl, 20ml/h. 5.0ml each fraction. The protein peak are poured, antibody activity are estimated with ELISA, active eluates are poured, adjusted to 20mg protein/ml, then, 1.5ml of it are applied on Sephadex G200 column (2.0x65cm), eluted with pH 7.0, 0.01M of phosphate buffer containing 0.1M of NaCl, 8.0ml/h. 5.0ml each fraction. The protein peaks are poured, estimated for antibody activity with ELISA, active eluates are poured, sterilized with 0.22p membrane, then lyophilized. This is purified IgY against dental caries bacteria.

Sample 2 Streptococcus mutans type c and type d are separately cultivated in TTY medium at 37°C for 48 hours, collected by centrifugation at 4000 rpm for 10 minutes, winsed with pH 6.5, 0.15M of phosphate buffered saline 5 times, heated at 65°C for 25 minutes, then make type c and type d suspensions, 2x10°C for 25 minutes, then, make type c and type d suspensions, 2x10/ml each, Mix equal volumes of type c and type d suspensions to get mixture (2x10 /ml) of them, Add Freund's adjuvant equal to the volume of the mixture, treat it with high speed homogenized to et streptococcus mutans antigens.

To get crude IgY extract against dental caries bacteria, immunize hens by three injunctions in wing vein, 1.0ml (1x10 /ml) of antigens each time, at 2 weeks intervals. Collect eggs from 20th day after first injuction, sterilize the eggs by 75% alcohol, take yolks out by sieve, stir them even, dilute with 6 fold volume of distilled water, adjust pH to 5.5, stand at 4°C for 24 hours, centrifuge at 8000 rpm for 25 minutes, concentrate the supernatant by ultra filtration, sterilize and lyophilize.

To get purified IgY against dental caries bacteria, apply 4.0ml (10 mg/ml) of crude IgY on Dear-Sephadex A50 column (2.5x35cm), elute with pH 7.0, 0.01M of

phosphate buffer containing 0.06M of NaCl, 20ml/h. 5.0ml each fraction; pour each peas, estimate antibody activity with ELISA. Keep the active eluates, sterilize by 0.22 p membrane and pyophilize.

Preparation of product of the combination preventing dental caries.

Sample 3 Preparation of IgY buccal liquid Take 2.0g of the IgY, 0.15g of potassium sorbate, 0.8g of sugar, and 0.15g of menthol and 0.4ml of apple essence. Add menthol into 100ml of distilled water and dissolved at 60°C, other solid components are dissolved in 450ml of distilled water, combine both solutions, then add distilled water to 1 000ml.

Sample 4 Preparation of chewing gum Take 2.0g of the IgY, 0.15g of potassium sorbate, 0.8g of sugar, 0.15g of menthol, 0.4ml of apple essence, 10.08g base and 5.08g of CM-cellulose, then ad substrate material to 1 000g.

Sample 5 Preparation of IgY toothpaste Take 0.1g of the IgY, 0.015g of potassium sorbate, 0.015g of sodium benzoate, 10.0g of glycerol, 8.0g of sorbitol, 2.0g of CM-cellulose, 1.3g of sodium trehalate, 1.8g of sodium lauryl sulfate, 0.015g of menthol, 0.015g of sugar, 0.05ml of strawberry essence, 47.8g of calcium phosphate. $2H_2O$. Swell CM-cellulose to dissolve followed by orderly adding other components, stir thoroughly, add distilled water to 1 000ml, and then stir until it becomes paste.

Sample 6 IgY tooth- protecting paste Take 0.1g of the IgY, 0.01g of sodium benzoate, 8.0g of beeswax, 10.0g of Stearic acid, 2.0g of monostearyl glyceride, 10.0g of glycerol, 1.0g of CM-cellulose, 0.01g of menthol, 0.05g of sugar, 68.80ml of distilled water and 0.02g of strawberry essence. Mix beeswax, Stearic acid, monotearyl glyceride and glycerol, and heat to 70°C, named solution A. Swell CM-cellulose in 50ml distilled water to dissolve, orderly and IgY, menthol, sugar, potassium sorbate, and streaberry essence, stir thoroughly, then add cooled solution a, add distilled water to 100ml, stir until it becomes paste.

Sample 7 Preparation of IgY nutrient milk Add IgY of the invention and potassium sorbate, whose final concentrations are 0.1% and 0.015%, respectively, into pasteurized frush milk, homogenize with sterile homogenized, pour into sterile sucking bottles, store at 4°C.

Sample 8 Preparation of IgY nutrient milk powder Take IgY of present invention and potassium sorbate, whose final concentrations are 0.1% and 0.005%, respectively, in pasteurized frush milk powder, mix with sterile mixed, package sterily in bags.

Sample 9 Preparation of IgY nutrient bean milk Add IgY of the invention and sodium benzoate, whose final concentrations are 0.1% and 0.05%, respectively in pasteurized compounded bean milk, homogenize with sterile homogenized, pour into sterile sucking bottles, store at 4°C.

Sample 10 Preparation of IgY nutrient bean milk powder Add IgY of the invention and sodium benzoate, whose final concentrations are 0.1% and 0.005%, respectively, in pastuerized bean milk powder, mix with sterile mixer, sterily package in bags.